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Direct somatic embryogenesis and plant regeneration of carnation (*Dianthus caryophyllus* L.)

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Abstract Conditions for efficient direct somatic embryogenesis and plant regeneration of leaf explants from carnation cultivars Lena (SIM group) and Bulgarian spray cultivars Nasslada, Yanita, Regina and Line 84 were established. Murashige and Skoog (MS) liquid medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.2 mg/l 6-benzylaminopurine was used for direct induction of embryoids without an additional callus phase. The first globular structures were observed after 20 days of cultivation. Their further development to the torpedo stage was correlated with the presence of polyethylene glycol (PEG 6000). Somatic embryo maturation was promoted by casein hydrolysate (1000 mg/l) in MS liquid media. The percentage conversion of embryos and polyembryos to whole plants varied between 10 and 75% among studied cultivars. Plantlets regenerated by this procedure were morphologically identical to the donor material and developed normally in a greenhouse.

Key words Carnation · Somatic embryogenesis · Regeneration

Abbreviations *MS medium* Murashige and Skoog medium · *2,4D* Dichlorophenoxyacetic acid · *PEG* Polyethylene glycol · *BAP* 6-Benzylaminopurine · *NPhA* N-1-Naphthylphthalamic acid · *NAA* 1-Naphthaleneacetic acid

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Introduction

Carnation (*Dianthus caryophyllus*) is one of the major floriculture crops in many countries of the world with high ornamental and commercial value (Burchi et al. 1996). In Bulgaria, 75% of the greenhouses destined for ornamentals are occupied by this crop. Modern cell and molecular techniques can be regarded as an alternative and complementary tool to the classical means for carnation improvement. However, their application requires the development of a routine and effective regeneration procedure.

Remarkable progress in in vitro regeneration of carnation was made recently, involving organogenesis from various explant sources (Frey and Janick 1991; Van Altvorst et al. 1992; Messeguer et al. 1993; Zuker et al. 1995). However, somatic embryogenesis is a preferred pathway, because it offers better potential for multiplication. It occurs either in dedifferentiated cells or directly in predetermined embryogenic cells (Ammirato 1985; De Jong et al. 1993). Induction of somatic embryos directly from plant tissue is the most desirable approach, because it appears to be associated with the cytological and genetic stability of regenerated plantlets (Vasil et al. 1988; Pedroso and Pais 1995). Efficient systems based on direct somatic embryogenesis have been developed for numerous species including alfalfa (Denchev et al. 1991), forest trees (Gupta et al. 1991; Merkle 1995), celery (Okamoto et al. 1994), red clover (Radionenko et al. 1994) *Camellia japonica* (Pedroso and Pais 1995), and chickpea (Barna and Wakhlu 1995).

Somatic embryogenesis in *D. caryophyllus* is not well documented. Protocols for indirect somatic embryo formation have been reported for a limited number of cultivars (Frey et al. 1992; Sankhla et al. 1995). Development of somatic embryos by culture of small white callus and directly on the leaf surface (Nakano and Mii 1993) has also been observed. However, in all mentioned cases, the regeneration efficiency was quite low.

In this study we describe a new procedure for direct somatic embryogenesis of economically important carnation

cultivars in liquid media. This system increased significantly the frequency of explant response and the number of embryos per responding explant in comparison to those obtained so far on solid and in liquid media.

Materials and methods

Plant material and culture conditions

Carnation cultivars Lena (SIM group) and Bulgarian spray carnation cultivars, Nasslada, Yanita, Regina and Line 84 were used in this study. The cultivars were grown at the Institute of Floriculture, Sofia. Virus-free nodal cuttings were used to establish an in vitro stock plant culture. They were surface sterilised for 20 min in 3% (vol/vol) solution of sodium hypochlorite (commercial bleach) and rinsed three times for 10 min with sterile distilled water. Plantlets were propagated in vitro by nodal cuttings every 30–35 days and maintained on hormone-free MS medium. In vitro donor material and suspension cultures were grown in a cultivation room at 22 °C, 16-h photoperiod, 1800 lux and 75% humidity.

Explant preparation

The first four leaf pairs collected from plants subcultured every 30–35 days were cut by razor blade into small pieces (1–4 mm^2) and rinsed once with sterile water, according to the procedure already described (Denchev et al. 1991). The initial leaf explants were inoculated into flasks (300 ml volume). The ratio between plant material and nutrient medium A was 1 : 1 (20 mg/l : 20 ml). Suspension cultures were maintained for 35 days on a rotary shaker (100 rpm).

Composition of the media

(1) Embryo induction: Murashige and Skoog (1962) (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4 D), 1 mg/l, 0.2 mg/l (BAP) and 2000 mg/l casein hydrolysate (medium A) was used. (2) Embryo development: somatic embryos and polyembryos rinsed with sterile water were cultured for 15 days in medium B [MS salts supplemented with polyethylene glycol (PEG, MW 6000) at concentrations of 0.5% (B_1), 0.75% (B_2), 1% (B_3), 2.5% (B_4), 5% (B_5)]. (3) Embryo maturation: three liquid media were applied: EM1 (MS with 1000 mg/l casein hydrolysate), EM2 (MS plus 1000 mg/l casein hydrolysate, 0.7 mg/l BAP), and EM3 [MS with 1000 mg/l casein hydrolysate, 0.5 mg/l BAP, 0.1 mg/l antiauxine N-1-naphthylphthalamic acid (NPhA). (4) Embryo conversion and rooting were performed on MSc [MS basal medium containing 1-naphthaleneacetic acid (NAA), 0.1 mg/l]. In all media, the pH was adjusted to 5.8 with 1 *N* NaOH before autoclaving.

Transplantation into soil

Ten “embryo regenerants” or each tested cultivar were analysed in a greenhouse. Plantlets were potted in a soil-perlite mixture (1 : 1) under mist conditions.

Histological analyses of the initiated somatic embryos

Leaf explants with initiated structures were fixed in formalin/acetic acid/alcohol (2 : 1 : 5 by volume) for 24 h. After paraffin embedding, the tissue was cut and stained with 1% toluidine blue for 30 s and observed microscopically.

Data analysis

Mean numbers of somatic embryos in different stages of development were counted in 10-ml samples of suspension culture. Presented results summarise the data of three independent experiments with three replicates in each run, which were evaluated by analysis of variance (ANOVA).

Results

Induction

All tested cultivars gave rise to somatic embryos after 20–25 days of cultivation in medium A when explants were excised from plants subcultured every 30–35 days. About 65% of the explants produced globular embryos during this period. If the size of explants was too small (~ 1 mm^2) they became brownish and failed to produce structures. The number of induced somatic embryos was remarkably low when the suspension culture was initiated from bigger leaf explants (half or one-third of the leaf).

Usually, the number of primary embryos ranged from 5 to 15 per explant and most of them appeared on the cut edges. They resembled clusters of embryo-like structures, which continued to develop for an additional 7–10 days without separation from the starting tissue. There was no significant difference in the number of somatic embryos produced among the studied cultivars. No deviation in their morphology was observed at this stage of development.

When the concentration of 2,4 D was increased to 4 mg/l, the induction period was reduced by 5 days. As a result, somatic embryos developed a more friable surface and were disposed to recallusing.

Development

Single globular embryos released in liquid media together with those still connected with the donor explants as polyembryo structures continued to develop to torpedo-stage embryos for 15 days in medium B. The data clearly demonstrated the positive effect of PEG at low concentrations. The best results were observed in medium B_3 (Fig. 1). The rate of somatic embryo development to torpedo stage in this medium was much higher than in B_1 and B_2 media. If the level of PEG was higher (B_4 and B_5) the differentiated structures turned brown and ceased development. Some differences in the colour and in the shape of torpedo somatic embryos between tested cultivars were observed. In the absence of PEG, globular structures usually turned to hard greenish callus.

Histological analyses of thin sections of leaf explants indicated that embryos were formed directly without a callus phase. They grew on the surface of the explants without any vascular connection with the maternal tissue (Fig. 2 a). Later on they were able to form their own anatomically independent vascular system (Fig. 2 b).

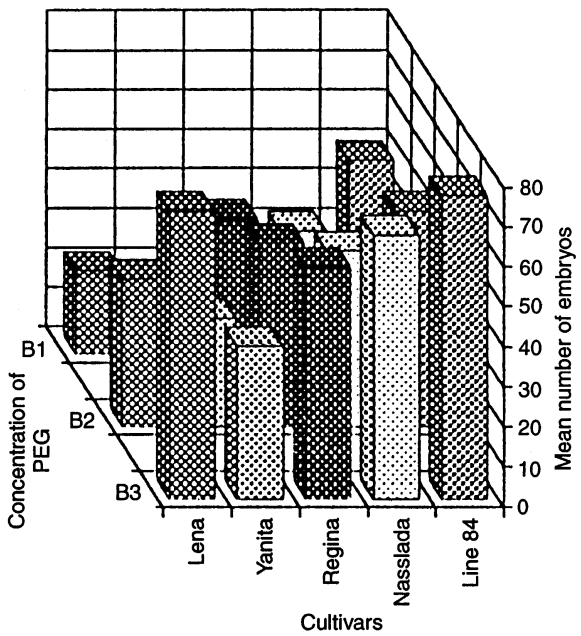

Fig. 1 Mean number of embryos developing to torpedo stage in medium B

Maturation

For a period of 2 weeks, part of torpedo embryos and polyembryos from cvs. Lena, Nasslada, Yanita and Line 84 developed their cotyledons in medium EM1 (Figs. 2 c, d, 3 a). Such embryos grew rapidly and rooted easily on media for conversion (MSc) (Table 1). In the presence of the cytokinine BAP at 0.7 mg/l (medium EM2), cv. Regina formed the highest number of somatic embryos, but most of their cotyledons remained undeveloped (Fig. 3 b). The efficiency of conversion of such structures to plantlets was relatively low (Table 1). In medium EM3 (Fig. 3 c), somatic embryos and polyembryos of all tested cultivars with the exception of cv. Regina often germinated precociously or gave rise to secondary embryos that could be induced repeatedly in this medium.

Table 1 Mean number of plants regenerated from somatic embryos on MSc after maturation on media EM1, EM2, EM3. Twenty embryos and polyembryos were plated in each Petri dish

Medium	Lena	Yanita	Nasslada	Regina	Line 84
EM1	33.66 ±3.21 [bc]	45 ±7.81 [bc]	180 ±82.6 [bc]	9 ±2.64 [b]	25 ±2.0 [b]
EM2	6 ±1.0 [a]	6.66 ±3.05 [a]	6.66 ±4.05 [a]	15.33 ±4.5 [ac]	9.66 ±0.57 [a]
EM3	10.66 ±1.1 [a]	19 ±7.93 [a]	7.33 ±3.78 [a]	8 ±2.64 [b]	5.33 ±0.88 [a]

[a] Significantly different from EM1 at $P<0.05$
[b] Significantly different from EM2 at $P<0.05$
[c] Significantly different from EM3 at $P<0.05$

Conversion and rooting

Randomly picked embryos and polyembryos at the cotyledonary stage were plated for 3 weeks on medium MSc. Significant differences were observed among the number of regenerants obtained after maturation in all liquid media used (Table 1). The highest conversion of somatic embryos was observed for cv. Nasslada (75%) followed by Yanita (45%), Lena (30%), Line 84 (25%) and Regina (10%). Ten regenerants of each tested cultivar were suc-

Table 2 Biometric analyses of tested plants. Data (in cm) are the mean ± SE (*R* regenerant, *C* control)

Plant morphology	Lena	Nasslada	Yanita	Regina	Line 84
Plant height	R 111±321 C 117±1.45	R 99±5.7 C 91±2.08	R 89±1.7 C 86±0.33	R 103±0.81 C 104±0.57	R 109±0.88 C 104±0.88
Leaf length	R 10.5±0.28 C 10.83±0.44	R 10.16±0.72 C 13±0.78	R 9.6±1.00 C 9.1±5.77	R 10.5±0.28 C 10.5±0.28	R 12.33±0.33 C 8.16±8.82
Leaf width	R 0.86±3.33 C 0.86±0.33	R 0.53±3.33 C 0.7±6.66	R 0.86±3.3 C 0.66±3.33	R 0.76±3.33 C 0.73±3.33	R 0.76±3.33 C 0.76±3.33
Number of internodes	R 19.66±0.66 C 19.33±0.33	R 18±1.15 C 18±0.57	R 19±0.57 C 17.66±0.33	R 20±0.57 C 18.3±0.33	R 19.33±0.33 C 19.66±0.66
Flower number	R 1±0 C 1±0	R 4.66±0.33 C 4.33±0.33	R 4.33+0.33 C 3.66±0.33	R 5.66±3.33 C 5.66±3.33	R 5±0 C 3.33±0.66
Flower stalk number	R 1±0 C 1±0	R 3.33±0.33 C 3.33±0.33	R 4±0 C 2.66±0.33	R 4.66±0.16 C 4.66±0.33	R 4.33±0.33 C 2.33±0.66
Diameter of flower cup	R 7.2±1.00 C 7.36±6.66	R 1.4±1.00 C 1.66±0.33	R 2.1±5.77 C 1.8±5.77	R 1.43±6.66 C 1.56±6.66	R 1.73±3.33 C 1.86±0.33
Diameter of flower	R 7.2±1.0 C 7.36±6.66	R 4.66±0.16 C 5.83±0.16	R 6.26±0.12 C 5.33±0.41	R 7.26±6.66 C 7.26±0.14	R 5.83±0.16 C 6.43±6.66
Height of flower cup	R 3.26±0.14 C 3.96±0.12	R 3.2±1.00 C 3.1±577	R 3.13±8.81 C 3.1±5.77	R 3.1±5.77 C 3.3±0.15	R 3.1±5.77 C 3.33±0.16
Height of flower	R 6.33±0.16 C 6.76±0.14	R 4.43±6.66 C 5.3±0.20	R 5.33±8.81 C 4.83±8.81	R 6.16±0.16 C 6.43±6.66	R 4.43±6.66 C 5.1±1.00

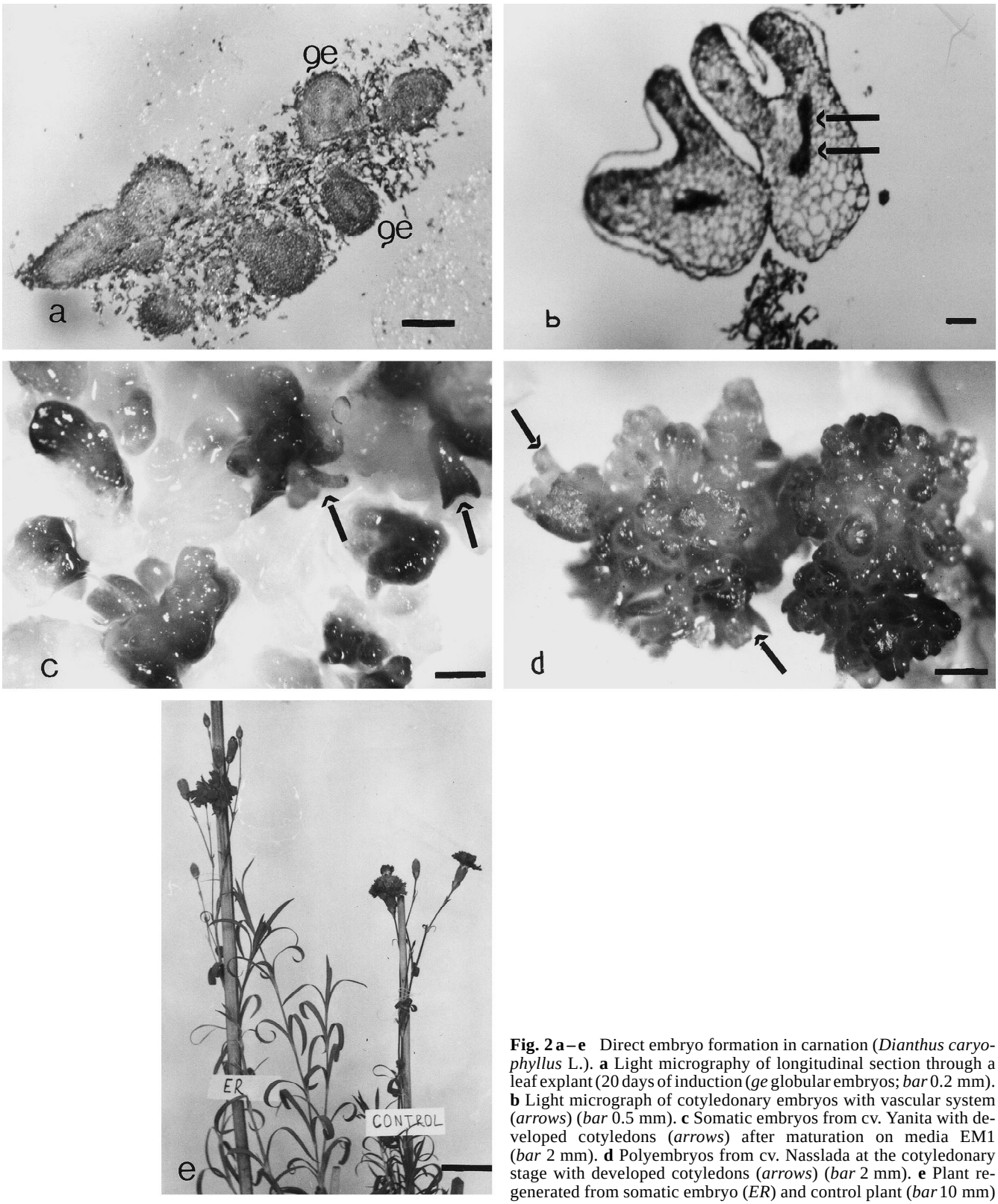

Fig. 2 a–e Direct embryo formation in carnation (*Dianthus caryophyllus* L.). **a** Light micrography of longitudinal section through a leaf explant (20 days of induction (*ge* globular embryos; *bar* 0.2 mm). **b** Light micrograph of cotyledonary embryos with vascular system (*arrows*) (*bar* 0.5 mm). **c** Somatic embryos from cv. Yanita with developed cotyledons (*arrows*) after maturation on media EM1 (*bar* 2 mm). **d** Polyembryos from cv. Nasslada at the cotyledonary stage with developed cotyledons (*arrows*) (*bar* 2 mm). **e** Plant regenerated from somatic embryo (*ER*) and control plant (*bar* 10 mm)

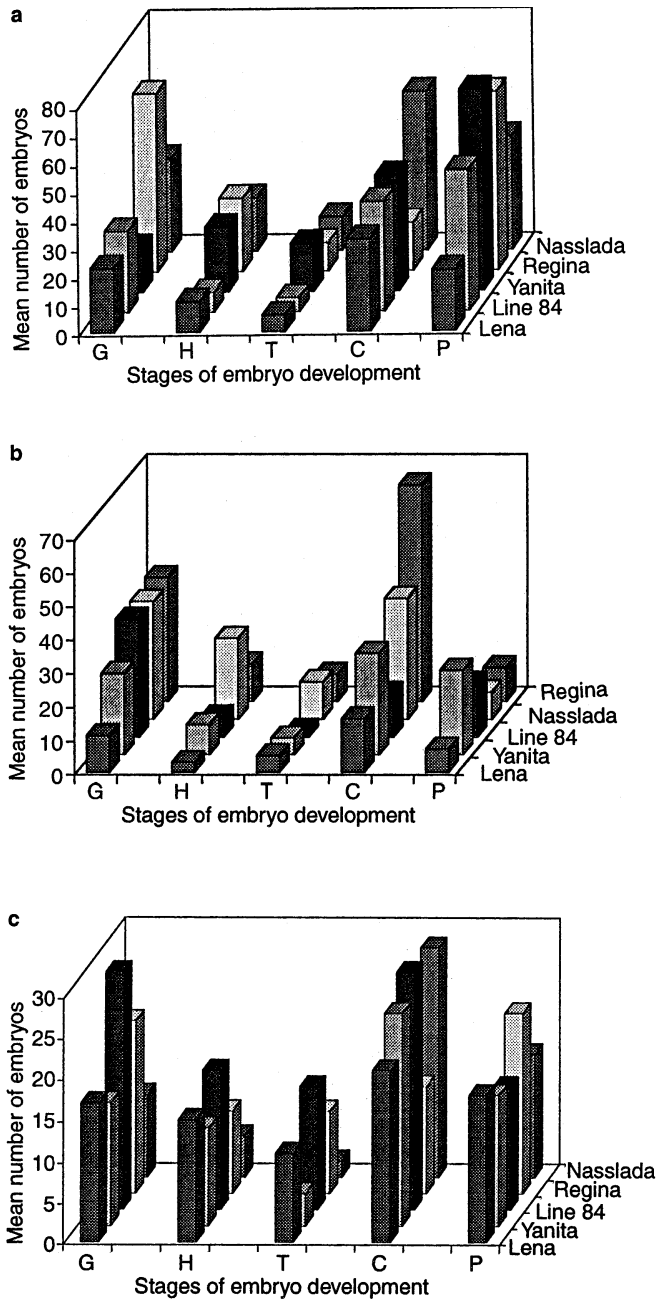

Fig. 3 Mean number of embryos in medium EM1 (**a**), EM2 (**b**) and EM3 (**c**) (*G* globular, *H* heart, *T* torpedo, *C* cotyledonany, *P* poly- and secondary embryos)

cessfully transferred and analysed in the greenhouse. They possessed the same phenotype as the control plants (Fig. 2, Table 2).

Discussion

In this study we have demonstrated the feasibility of obtaining a consistent response for direct somatic embryogenesis in liquid media for a number of commercially important cultivars. Unlike a previous observation (Frey et al. 1992), where some somatic embryos were induced from internodal callus in liquid media, in our procedure, a callus phase was avoided in order to obtain directly somatic embryos from leaf explants. Our results indicated that the previously published protocol for alfalfa direct somatic embryogenesis in liquid media (Denchev et al. 1991) after some modifications may offer a possibility for developing a routine procedure for direct somatic embryogenesis from leaf explants of carnation.

Leaves detached from in vitro plants subcultured every 30 – 35 days were the most suitable for our aims. This seems to correlate with the physiological state of the initial material. Preparation of the explants by razor blade and subsequent washing probably increases the embryogenic competence of wounded tissue due to changes at the level of endogenous growth regulators (Ivanova et al. 1994).

Among the tested auxins, 2,4 D (1, 2, 3 and 4 mg/l) strongly increased the embryogenic response of the leaf explants in all tested cultivars (data not shown). Normal growth and development of induced globular embryos was observed at 2,4 D concentrations below 2 mg/l. This phenomenon has already been observed in many other species (Phillips et al. 1994).

The development of the induced somatic embryos from globular up to torpedo stage was the main critical moment in this regeneration procedure. Embryo structures induced in unsuit able conditions could easily turn back to disorganised tissue. The addition of PEG as an osmoticum and dessicator played a very important role in carnation embryo development. In comparison to the published data for alfalfa (Denchev et al. 1991), a shorter period of treatment with PEG (15 days) and less osmotic pressure (B_3) were optimal for the carnation cultivars tested.

Under the applied conditions (EM1), the yield of cotyledonary embryos and developed plants for Lena, Yanita and Line 84 seemed not to be cultivar specific. In contrast, the number of regenerated plants from Nasslada was cultivar specific. Regina embryos required cytokinine-containing medium (EM2) to promote their maturation.

Only the combination of the antiauxin NPhA (0.1 mg/l) and BAP (0.5 mg/l) (medium EM3) provoked the formation of secondary embryos. These preliminary data for repetitive embryogenesis in tested cultivars show that it may be possible to develop a procedure for long-term maintenance of embryogenic potential in carnation.

Previous protocols for regeneration of carnation by somatic embryogenesis included a callus phase (Frey et al. 1992; Sankhla et al. 1995). Obviously, the above-described procedure is sufficiently efficient to avoid the callus phase which usually leads to genetic deviations. To our knowledge, this is the first report on direct somatic embryogenesis of carnation.

Conclusion

In this study we have demonstrated for the first time the feasibility of direct somatic embryogenesis of carnation in

liquid media. The established procedure offers some advantages: (1) protocol for a regeneration by direct somatic embryogenesis was developed for economically important cultivars, (2) plants regenerated from somatic embryos were adapted successfully to greenhouse conditions and possessed a normal phenotype and (3) the conditions established for secondary embryogenesis offer the possibility to use different methods for gene transfer and could form the basis for obtaining non-chimeric transgenic plants.

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